COMMUNICATION

Indolinone Derivatives Inhibit Constitutively Activated KIT Mutants and Kill Neoplastic Mast Cells

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Mastocytosis is a neoplastic disease caused at least in part by somatic mutations of the *c-KIT* proto-oncogene resulting in constitutive activation of its protein product, KIT, the receptor tyrosine kinase for stem cell factor. KIT stimulates mast cell proliferation and prevents apoptosis of neoplastic mast cells. To develop potential therapies for mastocytosis we used indolinones, small molecules that inhibit tyrosine kinases. Four indolinone derivatives (SU4984, SU6663, SU6577, and SU5614) inhibited wild-type KIT, but variably inhibited constitutively activated KIT mutants. SU4984, SU6577, and SU5614 were effective against KIT with juxtamembrane activating mutations, whereas only SU6577 could suppress KIT containing either juxtamembrane or kinase domain activating mutations. Furthermore, SU4984, SU6577, and SU5614 killed neoplastic mast cells expressing a juxtamembrane-mutated KIT, whereas SU4984 and SU6577 killed neoplastic mast cells expressing KIT bearing a kinase domain mutation. These data show a direct correlation between inhibition of constitutively activated KIT and the death of neoplastic mast cells, and point to specific tyrosine kinase inhibitors as a potential therapy aimed directly at a cause of mastocytosis. *Key words: c-KIT/mastocytosis/stem cell factor/tyrosine kinase inhibitor. J Invest Dermatol 114:392–394, 2000*

The proto-oncogene *c-KIT* encodes KIT (Yarden *et al*, 1987; Qiu *et al*, 1988), the receptor tyrosine kinase for stem cell factor (Martin *et al*, 1990), also known as mast cell growth factor. Somatic *c-KIT* mutations causing ligand-independent activation of KIT and cell transformation (Furitsu *et al*, 1993; Kitayama *et al*, 1995; Tsujimura *et al*, 1996; Hirota *et al*, 1998; Ma *et al*, 1999a) appear causal in certain types of mastocytosis (Nagata *et al*, 1995; Longley *et al*, 1996, 1999; Ma *et al*, 1999a).

Documented activating *c-KIT* mutations fall into two groups. One group consists of mutations in codon 816 of human *c-KIT*, or its equivalent positions in other species, resulting in single residue substitution for Asp816 in the receptor kinase domain. The other group of activating mutations includes single residue substitutions and in-frame insertions or deletions in the receptor intracellular juxtamembrane region, which disrupt inhibitory control on the receptor kinase activity exerted by a putative juxtamembrane α-helix (Ma *et al*, 1999b). All sporadic adult-onset mastocytosis patients examined to date, and a subset of pediatric cases with atypical clinical presentations, have activating codon 816 mutations (Longley *et al*, 1999), whereas activating juxtamembrane mutations are common in canine mastocytomas (Ma *et al*, 1999a) and in human gastrointestinal stromal tumors (Hirota *et al*, 1998).

In this report we show that inhibition of KIT by indolinone derivatives is sufficient to kill neoplastic mast cells. These results support a causal role for activating *c-KIT* mutations in the pathogenesis of mastocytosis and identify tyrosine kinase inhibitors as a novel therapy targeting a cause of mastocytosis.

MATERIALS AND METHODS

Compounds Indolinones are polycyclic compounds that bind the ATP binding pocket of receptor tyrosine kinases, inhibiting their activities (Mohammadi *et al*, 1997; Sun *et al*, 1998). Five indolinone derivatives were used in this study: 3-[4-Methyl-2-(2-oxo-1,2-dihydro-indol-3-ylidene-methyl)1*H*-pyrrol-3-yl]-propionic acid (SU5402), 4-[4-(2-Oxo-1,2-dihydro-indol-3-ylidenemethyl)-phenyl]-piperazine-1-carbaldehyde (SU4984), 3-[4-Methyl-5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1*H*-pyrrol-3-yl]-propionic acid (SU6663), 3-[5-(6-Methoxy-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-4-methyl-1*H*-pyrrol-3-yl]-propionic acid (SU6577), and 5-Chloro-3-(3,5-dimethyl-1*H*-pyrrol-2-ylmethylene)-1,3-dihydro-indol-2-one (SU5614). SU4984 has a relatively broad spectrum of inhibition, being effective against platelet-derived growth factor receptor, fibroblast growth factor receptor, and insulin receptor, whereas SU5402 effectively inhibits the fibroblast growth factor receptor (Mohammadi *et al*, 1997; Sun *et al*, 1998). SU5614 and SU6577 suppress vascular endothelial growth factor receptor and the platelet-derived growth factor receptor.[1] The potential activities of these compounds on KIT have not been previously reported.

Assays KIT activity was determined by *in vivo* phosphorylation, as previously described (Ma *et al*, 1999a). The neoplastic mast cell line C2 and P815 have been previously described (Dunn and Potter, 1957; Lazarus *et al*,

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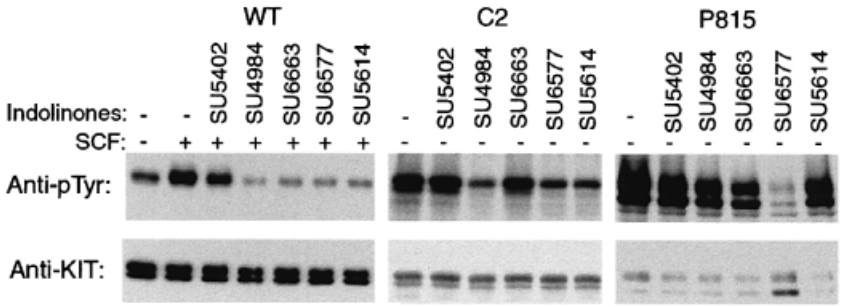

Figure 1. Effects of indolinone derivatives on phosphorylation of wild-type and mutant KITs. Upper panel: Anti-phosphotyrosine (pTyr) blots of immunoprecipitated wild-type (WT) KIT expressed in COS cells and of mutant KITs expressed in C2 and P815 cells stimulated (+), or not (–), with stem cell factor (SCF, 100 ng per ml, 10 min) after incubation (+), or not (–), with indolinone derivatives (5 μM for COS and C2 cells, 40 μM for P815 cells, 30 min) show that SU4984, SU6663, SU6577, and SU5614 inhibit wild-type KIT phosphorylation but variably inhibit C2 and P815 KIT phosphorylation. SU4984, SU6577, and SU5614 are effective against KIT containing an activating juxtamembrane domain mutation in C2 cells, whereas only SU6577 can significantly suppress KIT bearing an activating kinase domain mutation in P815 cells. Lower panel: Re-probing the anti-pTyr blots (after stripping) with anti-KIT antibody shows that comparable amounts of receptor are present in different lanes.

1986; DeVinney and Gold, 1990), and their activating *c-KIT* mutations characterized (Tsujimura *et al*, 1994; Ma *et al*, 1999a). To determine cell proliferation and viability, cells from triplicate cultures were stained with trypan blue and counted in a hemocytometer. All experiments were repeated at least three times.

RESULTS AND DISCUSSION

We first examined the effects of indolinone derivatives on the activity of wild-type KIT. SU4984, SU6663, SU6577, or SU5614 could all substantially reduce tyrosine phosphorylation of the wild-type receptor at 5 μM concentration, whereas SU5402 could only slightly decrease the receptor phosphorylation at this concentration (**Fig 1**).

We then tested the effects of these compounds on constitutive activation of KIT mutants expressed by neoplastic mast cells. For activating juxtamembrane mutations, we studied the C2 dog mastocytoma cell line (Lazarus *et al*, 1986; DeVinney and Gold, 1990), in which KIT is constitutively active due to a juxtamembrane insertion mutation (Ma *et al*, 1999a). Treatment of the cells with 5 μM of SU4984, SU6577, or SU5614 resulted in approximately 50% reduction of the constitutive C2 KIT phosphorylation (**Fig 1**). In contrast, 5 μM of SU5402 or SU6663 could barely repress the receptor phosphorylation. We also examined the effects of these compounds on constitutive phosphorylation of KIT bearing in the juxtamembrane region either deletion of Trp556-Lys557 or substitution of Pro for Leu575, mutations previously identified in dog mastocytomas (Ma *et al*, 1999a), and observed similar inhibitory effects on the mutant receptors (data not shown).

For activating mutations in the kinase domain, we examined the P815 murine mast cell tumor line (Dunn and Potter, 1957). P815 *c-KIT* contains a point mutation resulting in substitution of Tyr for Asp814, the equivalent position of human Asp816, which causes constitutive receptor activation (Tsujimura *et al*, 1994). Among the five compounds, only SU6577 at 40 μM concentration could substantially reduce the constitutive receptor phosphorylation (**Fig 1**).

To determine whether these compounds might be useful therapeutically for mast cell tumors, we examined their effects on C2 and P815 cell proliferation. As shown in **Fig 2**, C2 cells were killed by treating them daily with 1 μM of SU4984, SU6577, or SU5614. By comparison, SU5402 and SU6663 only retarded the C2 cell proliferation at this concentration. These results were consistent with the effects of the five compounds on the phosphorylation of KIT expressed by C2 cells (**Fig 1**).

The proliferation of P815 cells was not affected by treating the cells daily with the compounds at 1 μM concentration; however, SU4984 and SU6577 killed the P815 cells at 10 μM concentration, whereas SU5402, SU6663, and SU5614 showed only inhibition of

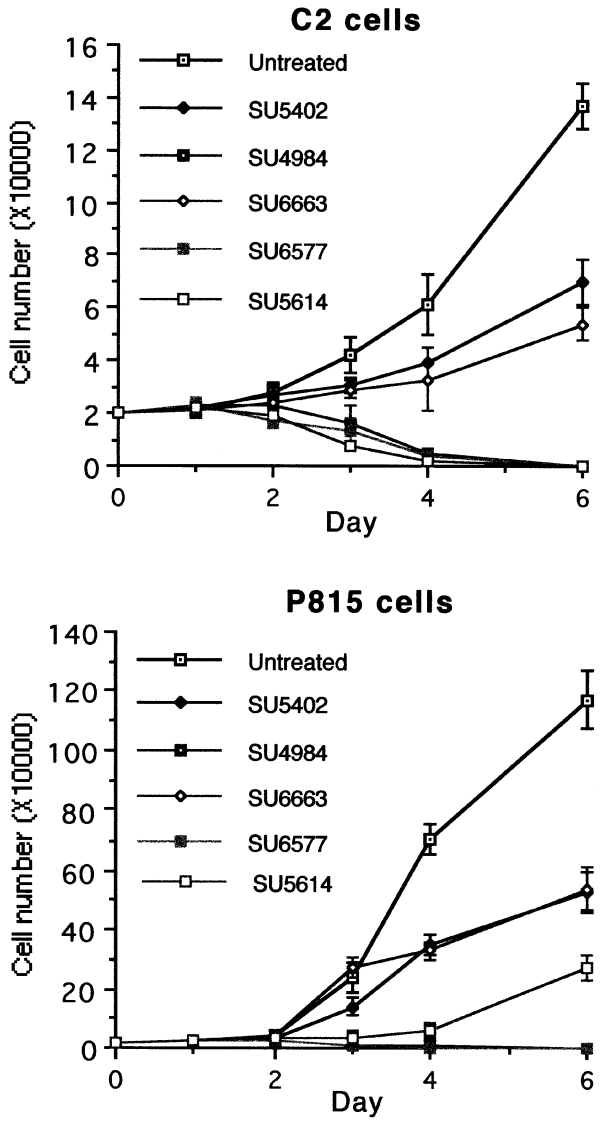

Figure 2. Effects of indolinone derivatives on neoplastic mast cell growth. Cell proliferation assay of C2 and P815 cells treated daily with 1 μM and 10 μM, respectively, of indolinone derivatives shows that SU4984, SU6577, and SU5614 kill C2 cells, and SU4984 and SU6577 kill P815 cells. Note that the growth curves of P815 cells treated with SU4984 and SU6577 overlap. Results (mean ± SEM) represent averages of triplicate cultures.

the cell proliferation at this concentration (**Fig 2**). The requirement of an order of magnitude of more SU4984 and SU6577 to kill P815 cells in comparison with their effects on C2 cells might be related to the difference in the *in vivo* behavior of these two cell lines. P815 cells are able to rapidly form metastasizing tumors in syngenic mice and kill the animals, whereas tumors formed by C2 cells in nude mice do not metastasize and do not kill the host.

Although SU4984 and SU6577 were both able to kill the P815 cells, they may exert their effects through different mechanisms. It has been reported that the mutation found in P815 cells alters KIT's substrate specificity (Piao *et al*, 1996). In this case, the relatively general inhibitor SU4984, although not effectively inhibiting phosphorylation of the KIT mutant, might still effectively repress other activating effector(s) in the KIT or other key oncogenic signaling pathway(s), resulting in the death of P815 cells. The fact that a compound that does not inhibit constitutively activated KIT can kill neoplastic mast cells does not detract from the theory that activating *c-KIT* mutations may be causal events in mastocytosis. Conversely, effective suppression of constitutively activated KIT is directly associated with the ability to kill neoplastic mast cells, supporting this hypothesis.

In summary, SU6577 effectively inhibited constitutively activated KIT bearing either juxtamembrane or kinase domain mutations and killed neoplastic mast cells expressing these mutants. Whereas the mechanism whereby SU4984 killed neoplastic P815 cells remains to be elucidated, this study identifies indolinone-derived tyrosine kinase inhibitors as the first potential therapy aimed at a cause of mastocytosis.

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